Binding and Internalization of Low-Density Lipoproteins in SCC25 Cells and SV40 Transformed Keratinocytes. A Morphologic Study*

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Binding of low-density lipoproteins (LDL) to the plasma membrane and internalization of low-density lipoprotein receptor complexes were investigated in an epithelial tumor cell derived from the tongue (SCC25) and in SV40-transformed keratinocytes (SVK14 cells). For light microscopic studies an immunofluorescence technique with antiapoprotein B as well as conjugation procedure by which a fluorochrome 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanide (DIL) was conjugated with LDL (LDL-DIL) was used. Binding of LDL to the plasma membrane at 4°C was observed in most SCC25 cells but not in SVK14 cells. The internalization of LDL-DIL was absent in SVK14 cells and was excessive in SCC25 cells. In SCC25 cells, internalization of the LDL-DIL particles was heterogeneously distributed over various cells. When a pulse-chase experiment was performed with LDL-DIL, less LDL was internalized into the SCC25 cells in comparison with a continuous label experiment. For the ultrastructural studies LDL conjugated with colloidal gold was used. In the binding experiments at 4°C most LDL-gold particles were attached to the plasma membrane outside coated pits. During internalization experiments with LDL-gold particles it was observed that within 5–15 min at 37°C several LDL-gold particles were seen in electron-dense structures near the plasma membrane.

The electron-dense structures containing LDL-gold, as observed after an internalization period of 5–15 min, may represent the first endosomal compartment as described for transferrin receptors in A431 cells.

After a period of 30 min at 37°C the LDL-gold particles were observed in electron-lucent vesicles (multivesicular bodies) and dense bodies. However coated vesicles containing LDL-gold particles were seen sporadically. It is concluded that the route of internalization of LDL into the SCC25 cells differs from that of other cell types.

No internalization of LDL gold was found in SVK14 cells, thus, in this respect, the SVK14 cells resemble normal keratinocytes.

The morphologic data are in good agreement with biochemical studies published earlier (Ponec M et al, J Invest Dermatol 83:436–440, 1984). Both investigations suggest that LDL receptor activity is modulated during the process of terminal differentiation. *J Invest Dermatol 86:195–200, 1986*

Many cell-biologic processes are regulated by ligand-receptor interactions [1,2]. One of these processes is cellular cholesterol synthesis which is regulated by low-density lipoproteins (LDL), the main cholesterol carrying protein in the bloodstream [3]. The LDL particles are first bound to plasma membrane receptors and subsequently the LDL-receptor complexes are internalized and the LDL are finally degraded in lysosomes. After degradation the LDL-cholesterol is released into the cytoplasm and exerts a down regulation of endogenous cholesterol and LDL receptor synthesis [4,5]. Morphologic studies with fibroblasts [6,7] have shown that most of LDL bound to the plasma membrane are present in coated pits. The receptor-mediated endocytosis of LDL uses the coated pit-vesicles system [8]. Many other ligand-receptor complexes [e.g., α_2-macroglobulin, epidermal growth factor (EGF), asialoglycoproteins] also use this system [9,10]. During the internalization process of ligand-receptor complexes, the receptors can be uncoupled and recirculate to the plasma membrane [11,12].

Contrary to most cell types described in the literature, no LDL receptor binding was observed by us in cultured keratinocytes [13]. Moreover, Anderson et al [14] and Gal et al [15] described a defective LDL metabolism in several epithelial tumor cell lines (e.g., A431, Ec168). Further studies revealed that the density of epithelial cell culture affected the LDL receptor metabolism [16,17].

In our biochemical experiments with epithelial tumor cells (e.g., SCC25, SCC15, SCC12F2, SCC12B2, and SCC4) a defective LDL metabolism was found which correlated with the degree of terminal differentiation of these cells [18]. In order to elucidate further the nature of the LDL receptor defects, morphologic studies on LDL binding and receptor-mediated endocytosis were performed on SCC25 cells. For comparison the same morphologic studies were performed on SV40-transformed keratinocytes (SVK14). These cells had practically no LDL metabolism according to our biochemical studies [18]. The morphologic data demonstrate that internalization of LDL in SCC25 cells occurs in a fashion similar to other epithelial tumor cells, but differs from SVK14 cells.

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Abbreviations:

DIL: 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanide
EGF: epidermal growth factor
LDL: low-density lipoproteins
PPDA: para-phenyl-ene-diamine
LPDS: lipoprotein-deficient serum

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MATERIALS AND METHODS

Lipoproteins Human LDL (density = 1.03–1.05 g/ml) were isolated from fresh serum of healthy individuals by density gradient ultracentrifugation followed by tube slicing. Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation (density > 1.25 g/ml) [19].

Cell Cultures Fibroblasts were cultured in Petri dishes containing Ham's F10 medium supplemented with 15% newborn calf serum. Human squamous carcinoma cells, SCC25 (derived from squamous cell carcinoma of the tongue) were kindly provided by Dr. J. Rheinwald and SV40-transformed keratinocytes (SVK14) were kindly provided by Dr. J. Taylor-Papadimitriou.

SCC25 cells (1×10^5) were plated together with 2×10^5 lethally irradiated 3T3 cells/7.5 cm^2 in Petri dishes containing a mixture of Dulbecco-Vogt's and Ham's F12 medium (3:1), 5% fetal calf serum, and 0.4 μg/ml hydrocortisone. SVK14 cells were cultured in the absence of the feeder layer in the medium of the same composition to which 10^{-6} M isoproterenol was added. After 1 week of culture the cells were not yet confluent and experimental procedures were started.

Conjugation of LDL For light microscopy studies, LDL were labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanide (DIL; molecular probes, LDL-DIL) according to Pitas et al [20].

For electron microscopy studies LDL were conjugated to colloidal gold (LDL-gold) as described by Handley et al [21]. In short, 0.5 ml of LDL (200 μg protein/ml) in 0.05 M EDTA at pH 5.5 was added to 5 ml of 20 nm colloidal gold and mixed by manual shaking. Unlabeled LDL were removed by centrifugation at 9000 *g* for 1 h against a 40% sucrose cushion. Each colloidal gold particle was surrounded by 6–8 LDL particles as checked by negative staining.

Specificity Antibodies The monospecificity of rabbit anti-human apoprotein B was checked by Ouchterlony double diffusion test and agar-immunoelectrophoresis [22].

Experimental Procedures Prior to incubations with LDL, the cells were always preconditioned for 24 h in medium containing 15% LPDS.

Binding and Endocytosis: For binding experiments, prechilled cells were incubated for 2 h at 4°C with unmodified or conjugated LDL (LDL-DIL or LDL-gold) at the concentration of 25–50 μg protein/ml. The endocytosis was studied by continuous incubation for 5, 15, or 30 min at 37°C with conjugated LDL (LDL-DIL or LDL-gold) at the concentration of 25–50 μg protein/ml.

Pulse-Chase Experiments: Pulse-chase experiments were performed by incubating cells for 2 h at 4°C with LDL-DIL at the concentration of 25 μg protein/ml. Then the cells were washed 3 times with phosphate-buffered saline, warmed up to 37°C, and incubated for 30 min at 37°C in medium containing 15% LPDS.

Visualization Procedures by Light Microscopy

Conjugation Technique: The cells incubated with LDL-DIL were fixed in 1% paraformaldehyde pH 7.4 for 30 min at room temperature, rinsed, and embedded in mounting fluid containing 0.1% para-phenyl-ene-diamine (PPDA).

Immunofluorescence Technique, Antiapoprotein B: After incubation with unmodified LDL, the cells were fixed in 1% paraformaldehyde pH 7.4 for 30 min at room temperature and a classical 2-step immunofluorescence technique was used; in the first step rabbit antihuman apoprotein B (1:20) and in the second goat antirabbit fluorescein isothiocyanate (1:80) (Nordic, Tilburg, The Netherlands) was applied. The cells were rinsed and embedded in mounting fluid containing 0.1% PPDA. For the controls, the cells were incubated either with normal rabbit serum instead of rabbit antihuman apoprotein B or with antihuman apoprotein B without preexposure to LDL.

Fluorescence Microscopy: The cells were viewed with a Leitz microscope with epifluorescent illumination using a rhodamine filter package for LDL-DIL and a fluorescence filter for immunofluorescence. Photographs were taken using a Vario XL Agfa film at 800 ASA.

Visualization Procedures by Electron Microscopy After incubation with colloidal gold-conjugated LDL particles, the cells were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 3 h at room temperature. After washing, the cells were incubated for 1 h in 0.005 M 3-amino-1,2,4-triazole (Sigma) in phosphate buffer and postfixed with 1% OsO_4 in phosphate buffer with 0.05 M sodium ferrocyanide for 1 h at room temperature [23].

Following dehydration in a graded ethanol series up to 70%, the cells were embedded in situ. Transversely and horizontally cut ultrathin sections were stained with lead hydroxyde and uranyl acetate and viewed with a Philips electron microscope 300 at 80 kV.

Control Experiments of LDL-Gold Particles The biologic activity of the LDL-gold particles was assessed in 2 control experiments. When cultured fibroblasts of a patient with homozygous familial hypercholesterolemia without LDL receptors were incubated with LDL-gold particles, no binding of these particles was observed. Moreover, the binding of LDL-gold particles at a concentration of 25 μg protein/ml was completely inhibited in normal fibroblasts by an excess of unlabeled LDL at a concentration of 200 μg protein/ml. When the cells (SCC25, SVK14) were exposed to pure colloidal gold, no attachment took place either.

RESULTS

Light Microscopy: Binding and Endocytosis of LDL When the SCC25 cells were incubated with unmodified LDL for 2 h at 4°C, the presence of apoprotein B was observed as brightly fluorescent stained clusters on the cell surface (Fig 1). The clusters were not arranged in a specific way. Under the same experimental conditions, only minimal deposits of apoprotein B were seen on the surface of SVK14 cells.

To visualize the internalization of LDL at the light microscopic level, the cells were incubated with LDL conjugated with the fluorochrome DIL. A continuous incubation of SCC25 cells for 5 min at 37°C with LDL-DIL resulted in the uptake of small fluorescent dots arranged throughout the whole cytoplasm (Fig

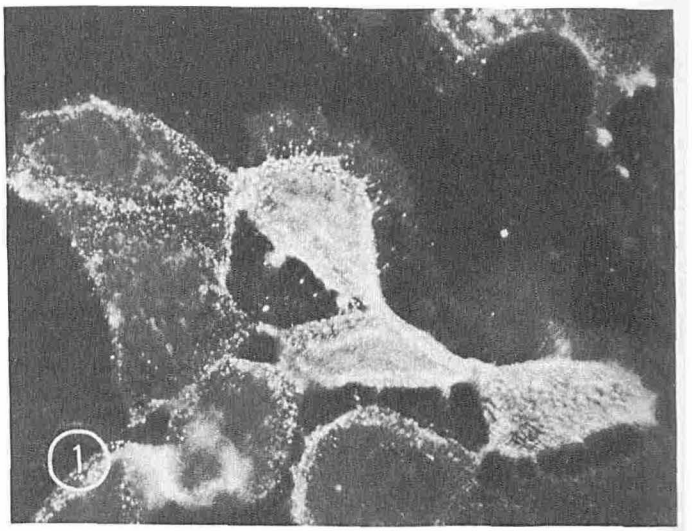
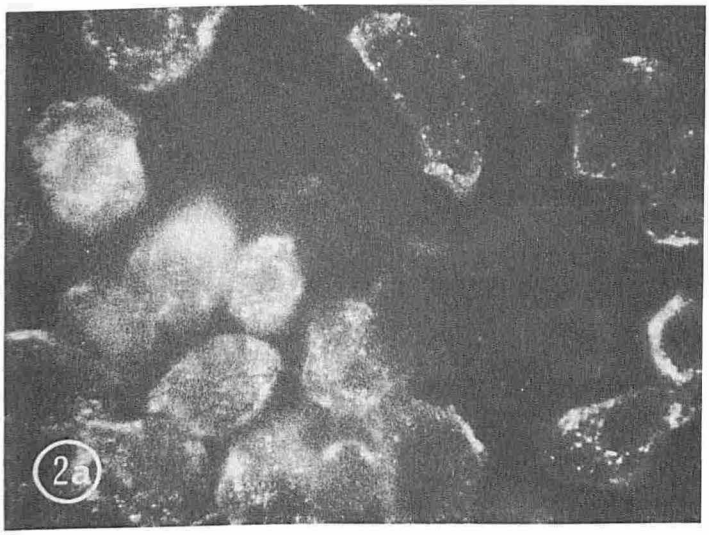
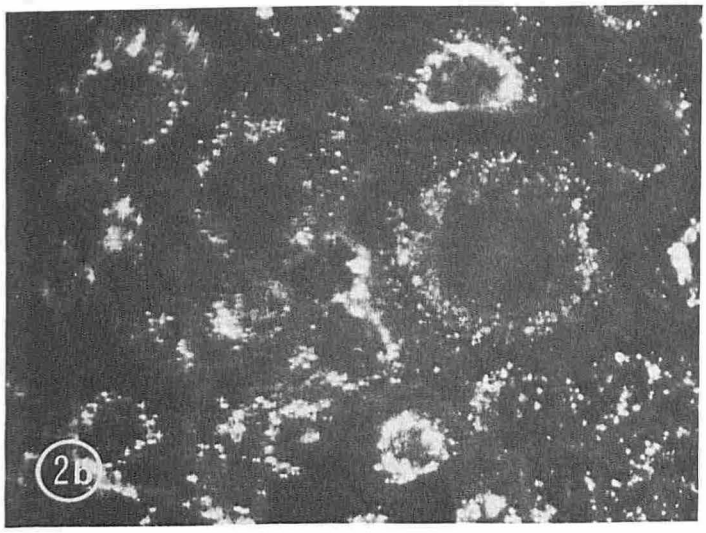


Figure 1. SCC25 cells incubated with LDL for 2 h at 4°C. Indirect immunofluorescence technique; LDL (apoprotein B) on the cellular surface. × 450.





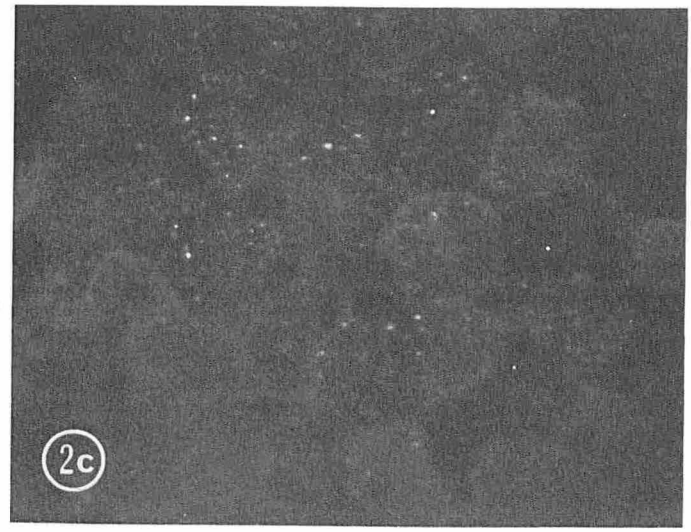


Figure 2. SCC25 cells incubated with LDL-DIL. × 450. *a*, Internalization of LDL-DIL for 5 min at 37°C, note small fluorescent dots. *b*, Internalization period of 30 min at 37°C, note perinuclear arrangement and heterogeneity of internalized LDL-DIL. *c*, Pulse-chase experiments, after binding of LDL-DIL for 2 h at 4°C, internalization of membrane bound LDL-DIL for 30 min at 37°C. Only some LDL-DIL particles are internalized.

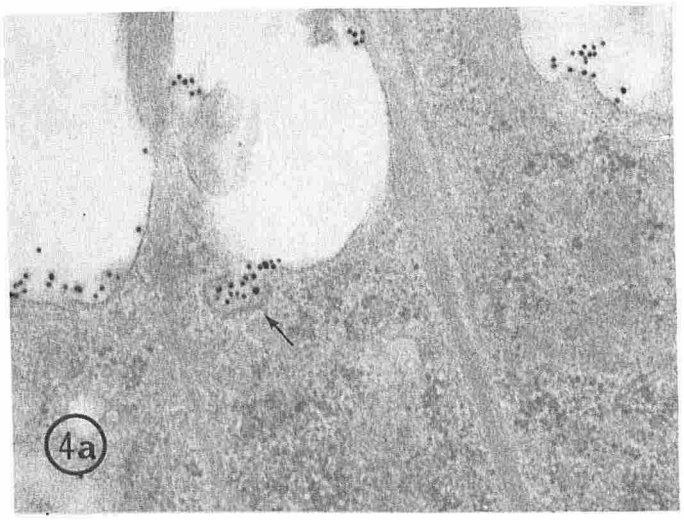
2*a*). When the cells were incubated for 30 min at 37°C with LDL-DIL the fluorescent markers were larger and seemed to be arranged in a perinuclear fashion. The nucleus is shown as round, dark-stained areas in the cell. It is noteworthy that several SCC25 cells internalized only few LDL-DIL particles during this period (Fig 2*b*).

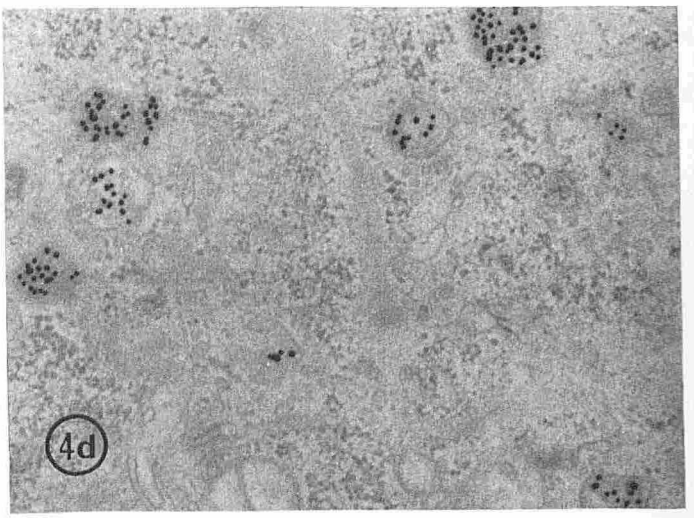
When LDL-DIL was first bound for 2 h at 4°C and thereafter incubated for 30 min at 37°C without LDL-DIL, internalization of surface-bound LDL-DIL took place (Fig 2*c*). In this pulse-chase experiment the amount of LDL-DIL internalized into the SCC25 cells seemed to be much lower, in comparison with the continuous incubation experiments. The internalization of LDL-DIL was minimal when SVK14 cells were incubated continuously for 30 min at 37°C with LDL-DIL (Fig 3). The latter observations could be made because the fluorescent labeled LDL enabled us to study larger surface areas of the culture.

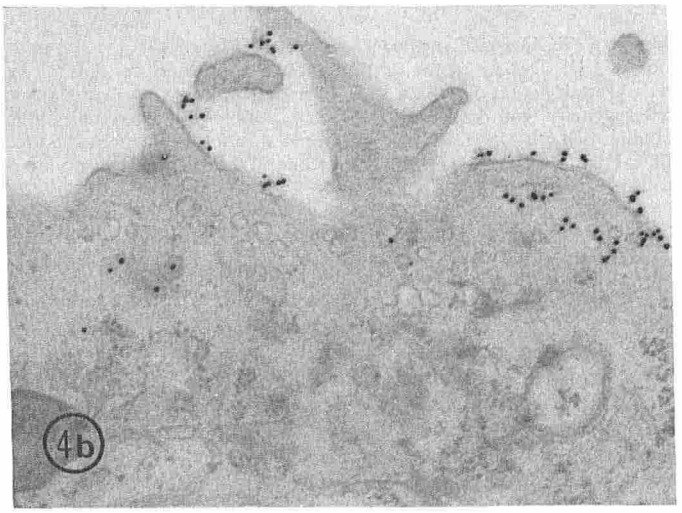
Electron Microscopy: Binding and Endocytosis of LDL-Gold When the SCC25 cells were incubated at 4°C with the low concentration of LDL-gold particles (25 μg LDL protein/ml) an excessive binding of LDL-gold particles was observed on the plasma membrane. A small number of LDL-gold particles were present in coated pits, whereas the majority of the particles were attached as clusters to noncoated regions of the plasma membrane (Fig 4*a*). The specificity of the LDL-gold binding was substantiated by experiments at 4°C, whereby an excess of unlabeled LDL (600 μg LDL protein/ml) was able to block most binding of LDL-gold particles (25 μg LDL protein/ml). Contrary to SCC25 cells, no binding of LDL-gold particles at 4°C was observed on the plasma membrane of SVK14 cells.

To investigate the internalization process the cells were continuously incubated with LDL-gold particles at 37°C. In the SCC25 cells a substantial amount of LDL-gold particles was rapidly internalized. After an incubation period of 5–15 min at 37°C, LDL-gold particles were present in small electron-dense structures near the plasma membrane (Fig 4*b*,*c*). When the SCC25 cells were incubated for 30 min at 37°C, LDL-gold particles were found in multivesicular bodies as well as in dense bodies (Fig 4*d*,*e*). When SVK14 cells were incubated for 30 min at 37°C, some LDL-gold particles were attached to the plasma membrane, but no internalization was observed (Fig 5).

Figure 3. SVK14 cells incubated with LDL-DIL for 30 min at 37°C. × 450. Compared with Fig 2*b*, only minimal internalization occurs.







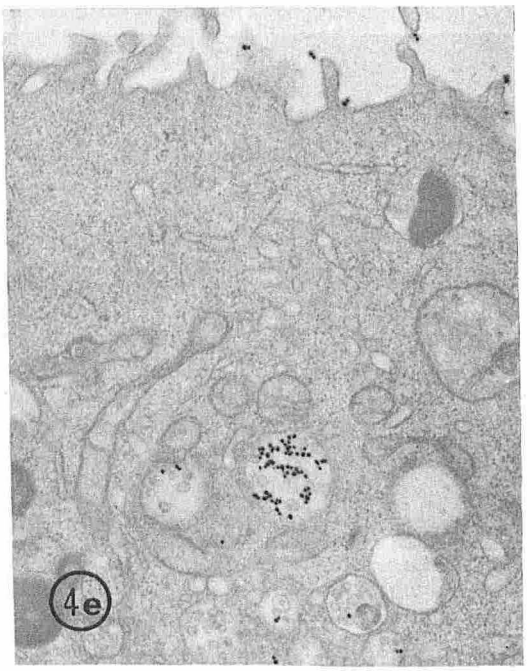


Figure 4. SCC25 cells incubated with LDL-gold 25 μg protein/ml. × 31,300. *a*, Two hours at 4°C, LDL-gold particles are bound in abundance to the plasma membrane, *arrow*; LDL-gold particles in coated pit. *b*, Five minutes at 37°C, various small electron-dense structures containing LDL-gold. *c*, Fifteen minutes at 37°C, many LDL-gold particles are present in small electron-dense structures. *d and e*, Thirty minutes at 37°C, many LDL-gold particles are present in dense bodies and in electron-lucent vacuoles (multivesicular bodies).

DISCUSSION

Various morphologic techniques demonstrated an excessive binding of LDL at 4°C to the surface of SCC25 cells (Figs 1, 4*a*). However, in contrast with fibroblasts, the binding of LDL to the surface of SCC25 cells was not linearly arranged [24]. Moreover, the ultrastructural studies showed that most LDL-gold particles were present as clusters outside specific membrane regions (e.g., coated pits). However, when in parallel experiments fibroblasts or endothelial cells were incubated with the same LDL-gold suspension, no LDL-gold clusters occurred. These data suggest that the LDL-gold clusters are specific and not due to self-aggregation in the LDL-gold suspension. Using LDL-ferritin particles, the same type of LDL distribution has been described for other epithelial tumor cells (A431, EC168) [14,15].

Whereas no LDL binding to SVK14 cells was observed at 4°C some binding was present when the same cells were incubated with LDL-gold at 37°C. This discrepancy may be explained by the increased binding capacity of plasma membrane receptors at higher temperatures. A similar phenomenon was described for the binding of apoprotein C III to nonparenchymal liver cells [25]

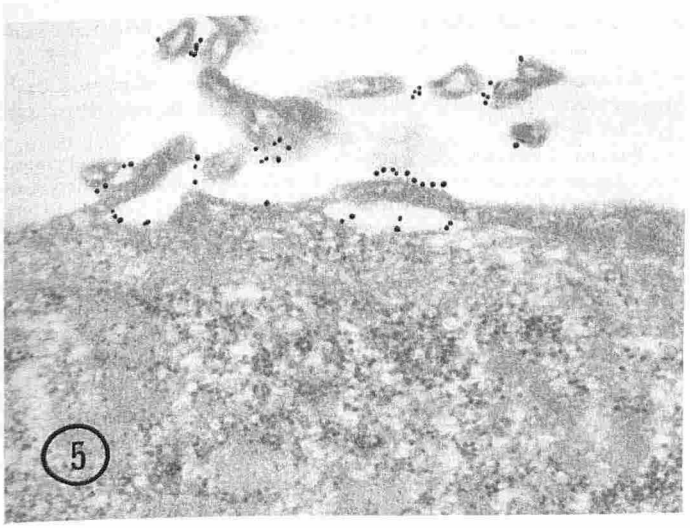


Figure 5. SVK14 cells incubated for 30 min at 37°C with LDL-gold 25 μg protein/ml. × 31,300. Only some LDL-gold particles are bound to the cell surface.

and of LDL to monocyte-derived macrophages [26]. Nevertheless, in spite of the temperature of 37°C the SVK14 cells did not internalize the LDL-gold particles, and at the light microscopic level in a few SVK14 cells minimal uptake of LDL-DIL particles was found.

In contrast to SVK14 cells, the internalization of LDL by the SCC25 cells was substantial. The distribution of LDL-DIL particles after an incubation period of 5–30 min at 37°C was the same as described by Dickson et al [27] for EGF-receptors in K13 carcinoma cells. In contrast with the continuous incubation experiment with LDL-DIL for 30 min at 37°C (Fig 2*b*) only small amounts of LDL-DIL were internalized when a pulse-chase experiment was performed (Fig 2*c*). This difference can be explained by the fact that during the continuous incubation period LDL-receptors, after internalization, are uncoupled and recirculated to the plasma membrane, and can therefore internalize the LDL still present in the medium [28]. However, the exact location of the uncoupling compartment of the LDL receptor is not yet known. With recently developed techniques, Geuze et al [11] could at the ultrastructural level demonstrate that in hepatocytes the uncoupling compartment for asialoglycoproteins resides in a special compartment called compartment of uncoupling receptor ligand. In our ultrastructural studies, a continuous labeling experiment with LDL-gold particles was performed. When the SCC25 cells were incubated for 5–15 min with LDL-gold particles, several particles were observed in small electron-dense structures near the plasma membrane. Hopkins and Tronbridge [29] described the same type of distribution for transferrin receptors in A431 cells. According to the authors, these electron-dense structures represent branching cisternae and form the first endosomal compartment. It is conceivable that these branching cisternae are a characteristic phenomenon in various cells (e.g., epithelial tumor cells).

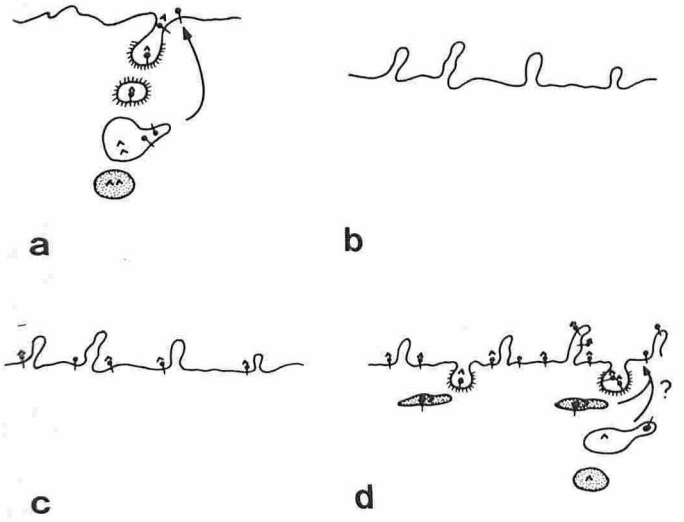


Figure 6. Binding and internalization of LDL-gold particles are schematically shown: ↑ = LDL receptor; ∧ = LDL. *a*, Fibroblasts; internalization of LDL, at 37°C; the receptors are uncoupled and recirculate to the cellular surface. *b*, SVK14 cells; no binding of LDL occurs at 4°C. *c*, SVK14 cells; binding but no internalization of LDL occurs at 37°C. *d*, SCC25 cells; binding and internalization of LDL at 37°C; characteristic electron-dense tubular organelle contains LDL. The presumptive pathway of LDL-receptor recirculation is indicated by *arrow*.

Moreover, we observed in SCC25 cells only sporadic coated vesicles containing LDL-gold particles. This observation differs from published data on the internalization of LDL into fibroblasts, A431 cells, and endothelial cells [14,15,30,31].

When the internalization of LDL-gold particles by SCC25 cells was studied for 30 min at 37°C, most particles were present in dense bodies and electron-lucent vacuoles containing small vesicles (multivesicular bodies). This localization was similar to the internalization of LDL by various other cell types (e.g., fibroblasts, endothelial cells, A431 cells) [8,14,15,30,31].

The differences in LDL binding and uptake between various cell types (e.g., fibroblasts, SVK14 cells at 4°C and 37°C, SCC25 cells) are schematically shown in Fig 6.

To elucidate further the pathway of receptor recycling, immunoelectron microscopic studies with receptor antibodies are needed.

The morphologic observations described in this paper are well in accordance with our biochemical experiments using [^{125}I]-LDL, as described earlier [18]. These studies demonstrated that no LDL receptor activity was present in SVK14 cells whereas the LDL receptor activity of SCC25 cells was impaired, compared with fibroblasts [18]. Moreover, the cellular cholesterol synthesis in SVK14 cells was much higher than in SCC25 cells or fibroblasts [18]. This phenomenon might imply an elevated cholesterol content of the plasma membrane and hence an increase in membrane rigidity and decrease of membrane receptor activity. Interestingly, the defect in LDL receptor metabolism in the different epithelial tumor cells correlated with defectiveness of the terminal differentiation as observed by cross-linked envelope formation after ionophore treatment [32].

The heterogeneous distribution of LDL internalization in SCC25 cells as shown in Fig 2*b* could also be explained by the fact that in the cell culture the cell population is not necessarily homogeneous and various cells differ in their degree of terminal differentiation.

The decrease of LDL receptor activity during the process of terminal differentiation is currently under investigation. We could recently demonstrate that LDL receptor activity of an epithelial tumor cell line was excessive when cultured at low density and nearly no terminal differentiation was present. However a decrease of LDL receptor activity took place when the cell density was high and terminal differentiation was present [33]. A similar phenomenon was observed when keratinocytes cultured in medium with a low calcium content (no terminal differentiation) showed LDL receptor activity, whereas the LDL receptor activity was absent if terminal differentiation was present in keratinocytes cultured in normal calcium content [13,34].

The findings of O'Keefe and Payne [35] and Boonstra et al [36] that EGF receptor activity also decreased in keratinocytes and epithelial tumor cells during the process of terminal differentiation favor the hypothesis that the modulation of receptor activity during terminal differentiation is a more general phenomenon.

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